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Genetic Typing of the Porin Protein of *Neisseria gonorrhoeae* from Clinical Noncultured Samples for Strain Characterization and Identification of Mixed Gonococcal Infections

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Molecular methods that characterize the *Neisseria gonorrhoeae* porin protein Por are needed to study gonococcal pathogenesis in the natural host and to classify strains from direct clinical samples used with nucleic acid amplification-based tests. We have defined the capabilities of *por* variable region (VR) typing and determined suitable conditions to apply the method to direct clinical specimens. Nested PCR from spiked urine samples detected 1 to 10 copies of template DNA; freezing spiked whole urine greatly reduced the ability to amplify *porB*. In a laboratory model of mixed gonococcal infections, the *por* type of one strain could be determined in the presence of a 100-fold excess of another. *por* VR typing was used to examine clinical samples from women enrolled in studies conducted in Baltimore, Md., and Madagascar. *por* type was determined from 100% of paired cervical swab and wick samples from 20 culture-positive women from Baltimore; results for eight individuals (40%) suggested infection with more than one strain. In frozen urine samples from Madagascar, *porB* was amplified and typed from 60 of 126 samples from ligase chain reaction (LCR)-positive women and 3 samples from LCR-negative women. The *por* VR types of 13 samples (21%) suggested the presence of more than one gonococcal strain. Five *por* types, identified in >45% of women with typed samples, were common to both geographic areas. Molecular typing is an important adjunct to nucleic acid amplification-based diagnostics. Methods that utilize direct clinical samples and can identify mixed infections may contribute significantly to studies of host immunity, gonococcal epidemiology, and pathogenesis.

Reproductive tract infections caused by *Neisseria gonorrhoeae* (gonococci) remain a significant public health issue throughout the world. Not only do gonococci cause uncomplicated gonorrhea, but infection can also lead to disseminated disease, pelvic inflammatory disease, or salpingitis leading to infertility, ectopic pregnancy, and chronic pain. Surveillance by the Centers for Disease Control and Prevention indicated that 361,705 cases of gonorrhea were reported in 2001 in the United States (5), and the World Health Organization estimated that there were 62.35 million cases worldwide in 1999 (34).

The development and increased use of nucleic acid amplification tests (NAAT) have had broad implications for the diagnosis and characterization of gonococcal infections. NAAT are more sensitive than culture (32) and are used to test noncultured samples such as urine, vaginal swabs, and tampons (15, 23, 26). Therefore, NAAT can be used to easily screen individuals, including those who are asymptomatic, without the need for a physical exam.

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Reliance on bacterial cultures for characterization of infecting strains is less feasible with increased use of newer molecular diagnostic methods. A parallel evolution in molecular approaches is needed to characterize strains for epidemiologic, public health, and research purposes such as identification of high-frequency transmitters and linked infections and studies of transmission and spread of resistance (4, 10). The use of NAAT has already opened new avenues of investigation; it has detected organisms from culture-negative sexually transmitted infections and identified the presence of multiple strains (16, 18). As *N. gonorrhoeae* is an obligate human pathogen which is highly diverse, with extensive molecular mechanisms for genetic exchange and recombination (13), it is likely that genetic exchanges take place when more than one strain infects an individual host. However, multiple infecting strains have been infrequently recovered from cultures, and thus, new molecular methods are needed that can reliably identify and characterize multiple individual strains in direct noncultured clinical samples.

The porin protein (Por), the major outer membrane protein of *N. gonorrhoeae*, is essential for bacterial viability, with all strains expressing one of two mutually exclusive alleles, PIA and PIB. Por is not phase variable and has been shown to be an important factor in the pathogenicity of the organism; it has been implicated in invasion in vitro (7, 11), and invasive dis-

ease is associated with strains expressing a PIA allele (25). Por has also been shown to contribute to serum resistance by binding complement regulatory proteins (22). Por is encoded by a single-copy gene, *porB*, and antigenic diversity within each allele has been localized to the eight predicted surface-exposed loops encoded by *porB* variable regions (VR) (30). We have previously described a genetic method for characterization of Por in gonococci. This method uses oligonucleotide probes with DNA-DNA hybridization to identify sequences of the VR, i.e., the sequences coding for the putative surface-exposed loops. The method characterizes sequences coding loops 1, 2, 3, 6, and 7 of the PIA porin and loops 1, 3, 5, 6, and 7 of the PIB porin based on determinations of the genetic regions that exhibit the highest levels of diversity (19, 27). Characterization of Por diversity remains an important factor in the evaluation of Por as a vaccine candidate.

In this study, we have adapted *por* VR typing for use with non-culture-based clinical samples. We report on the ability and limitations of our method to characterize *porB* from cervical swab, cervical wick, and urine samples. We also report on the diversity of *por* types and evidence suggesting that there are mixed gonococcal infections detected in archived samples from women from two divergent populations.

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METHODS AND MATERIALS

DNA preparation. (i) Bacterial strains. Strains used as *por* VR typing controls have been previously described (19). Strains MS11mkC, FA1090, FA19, and PI83 were used in spiked urine and mixed-strain experimental samples. Genomic DNA for *por* VR typing controls was isolated from cultures grown overnight on standard gonococcal agar plates by using a Wizard Genomic DNA purification kit (Promega, Madison, Wis.). Genomic DNA from the following *Neisseria* species was kindly provided by P. Zhu (Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md.): *N. meningitidis* (strain H335), *N. polysaccharea*, *N. lactamica*, *N. subflava*, *N. canis*, *N. cinerea*, *N. caviae*, *N. elongata*, *N. flavescens*, *N. mucosa*, *N. ovis*, *N. sicca*, and *N. weaveri* (35).

(ii) Experimental samples. Control urine was obtained from healthy volunteers and spiked with various dilutions of bacterial suspensions. Bacteria were prepared from strains grown as described above and harvested into Dulbecco's phosphate-buffered saline with magnesium, calcium, and 0.1% gelatin diluted to an optical density at 600 nm of approximately 0.1. Bacterial suspensions (CFU per milliliter) were determined by colony counts on standard gonococcal plates; the number of copies of template DNA was calculated based on the assumption that each CFU contained two bacteria and, thus, two copies of porin DNA. One-milliliter aliquots of spiked urine were centrifuged in a standard tabletop centrifuge (Eppendorf 5415C) at 14,000 rpm for 10 min, and DNA was isolated by using a High Pure DNA template kit (Roche Diagnostics Corporation, Indianapolis, Ind.) according to the manufacturer's instructions.

For evaluation of the effect of a freeze-thaw cycle on the ability to amplify *porB* from urine, bacterial suspensions were added to control urine, aliquoted, and subjected to various storage conditions. One set of aliquots was frozen, thawed, and centrifuged at the time of use (frozen urine); a second set was centrifuged, the supernatant was removed, and the pellet was stored frozen (frozen pellet); and a third set was centrifuged and processed immediately (fresh). DNA was isolated by using a Roche High Pure DNA template kit as described above.

To examine the ability of *por* VR typing to identify a mixture of different strains, suspensions of PIA and PIB control strains were prepared. CFU determinations were performed to confirm equivalent CFU per milliliter for each strain suspension (Fig. 1). PIA and PIB suspensions were then diluted 1:10 and 1:100, and these dilutions were combined with the undiluted suspensions to give ratios of PIA to PIB (CFU:CFU) of approximately 100:100, 100:10, 100:1, 1:100, and 10:100. One milliliter of each of the mixtures was centrifuged and processed for DNA as described above for the urine samples.

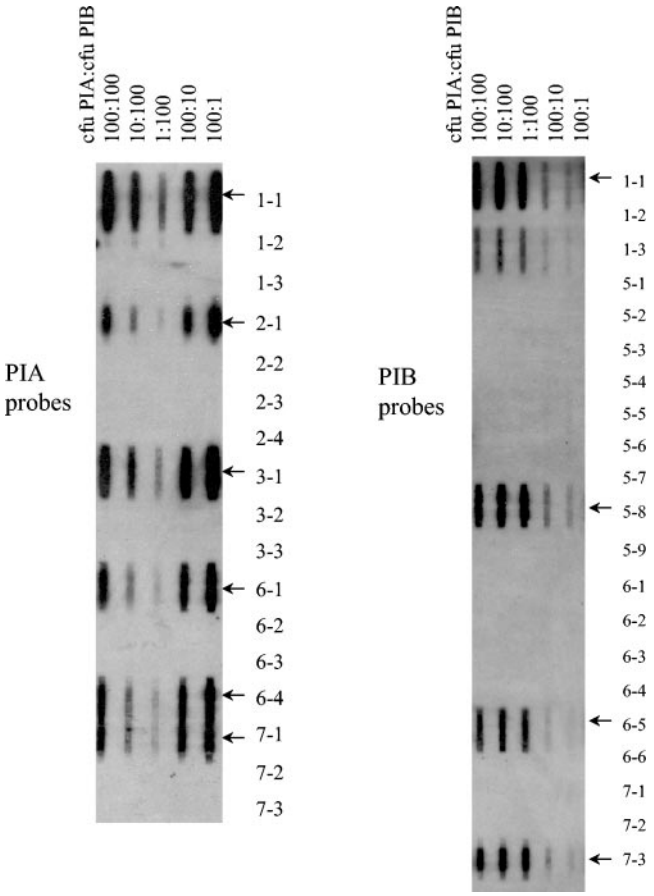

FIG. 1. *por* VR typing of PCR-amplified DNA from mixtures of two bacterial suspensions using checkerboard hybridization. Suspensions of PIB and PIA strains were mixed at different ratios, centrifuged, and DNA purified, and *porB* was PCR amplified. PCR product was applied to the nylon membrane in columns on the basis of total amplicon concentration; approximate CFU-to-CFU ratios shown, 100, 10, and 1, are equal to approximately 10^4, 10^3, and 10^2 CFU added to the PCR, respectively. Biotin-labeled probes were hybridized in rows, and probes that determine VR type are indicated by arrows. Shown are two images of the same blot initially probed with PIA probes and stripped and subsequently rehybridized with PIB probes. Both PIA and PIB strains could be detected by *por* VR typing in the presence of a 100-fold-higher concentration of another strain. Probe PIB VR1-3 is known to cross-react with VR1-1 (2-bp difference) based on comparison to control strains (data not shown), and its binding does not indicate a mixed *por* type.

(iii) Clinical samples. Samples from two studies were obtained to evaluate *por* VR typing from clinical specimens. In one study, subjects were a systematic sample of patients presenting to Baltimore sexually transmitted disease clinics from 1995 to 1996 (the Mucosal Immunology Study). For women who consented, cervical culture for gonococci was performed, and cervical swab, cervical wick (Sno-strip; Chauvin Pharmaceuticals Ltd., Essex, England), urine, and serum samples were collected. Swabs and wicks were stored in phosphate-buffered saline at −70°C. For positive cultures, a sweep of all growth was harvested from the primary plate and stored at −70°C in 20% glycerol–tryptic soy broth. We evaluated matched swabs and wicks from 20 subjects who were positive for *N. gonorrhoeae* by culture and did not participate in subsequent visits of the original study. The corresponding primary cultures, available for 17 participants, were also evaluated. DNA was purified from approximately half of the volume of buffer used to store the wick or swab (150 and 500 μl, respectively) or approximately 200 to 250 μl of frozen culture stock. Samples were centrifuged, the

pellets were treated at 56°C for 30 min, and DNA was purified as described above for the urine samples.

The second set of samples was first-void urine specimens collected as part of a study designed to develop sexually transmitted infection control strategies for female sex workers (SWs) in Madagascar which was conducted between May 2000 and January 2001 (2). Women provided specimens upon initial enrollment and received presumptive treatment for gonorrhea and *Chlamydia* infection (1 g of azythromycin and 500 mg of ciprofloxacin). A second specimen was collected approximately 2 months later. Specimens were aliquoted into two tubes on site, frozen at −20°C, and shipped on dry ice to the University of North Carolina, where one aliquot was tested by ligase chain reaction (LCR) (LCx probe system; Abbott Laboratories, Abbott Park, Ill.) and the other 0.1- to 5.0-ml aliquot was stored at −70°C. A total of 393 urine samples from 269 subjects were available. Urine samples ≥2 ml from both visits were available for 127 SWs, and 144 samples from the first 72 of these were arbitrarily chosen for *por* VR typing blinded to LCR results as a pilot study to examine the utility of our methods. An additional 31 of 41 LCR-positive samples that were 4 ml or greater were also tested. Samples were thawed and centrifuged, and the pellets were treated at 56°C for 30 min and then DNA purified by using a Roche High Pure DNA template kit as described above.

All studies were approved or exempted by the appropriate institutional review committees at Johns Hopkins University, the University of North Carolina, and the Center for Biologics Evaluation and Research.

DNA amplification. PCR amplifications were performed as previously described (19) in 50-μl reactions, using 10 μl of template DNA for experimental and noncultured samples or 1 μl of template DNA from gonococcal cultures, with 1 μmol of each primer, 300 μmol of deoxynucleoside triphosphate, 5 μl of 10× PCR buffer with $MgCl_2$, and 3.3 U of DNA polymerase with proofreading enzyme (Expand High Fidelity PCR system; Roche). PCR for all samples was performed by using primers GCPorBFouter (5′-TCGGCGGTAAATGCAAAGC-3′) and GCPorBRouter (5′-TGCAGATTAGAATTTGTGGCG-3′). Nested PCR was performed for the sensitivity determinations, the nongonococcal *Neisseria* species, and non-culture-based clinical samples by using primers PIB.Fpr (5′-ATTGCCCTGACTTTGGCAGCCCTTCCT-3′) and PIB.Rpr (5′-TTGCAACCAGCCGGCAGAAACCAAGGC-3′) (27) and 10 μl of the outer reaction as template. Both sets of primers amplify both PIA and PIB *porB*. Product concentration and size were determined by using the Agilent (Rockville, Md.) 2100 Bioanalyzer according to manufacturer's instructions. Reactions using distilled water instead of template DNA served as negative controls for each set of PCRs.

***por* VR typing.** The biotin-labeled oligonucleotide probes matching coding regions for the variable regions of *porB* have been described previously (19, 27). Probes for PIA VRs 1, 2, 3, 6, and 7 (three, four, three, four, and three probes for each VR, respectively), and PIB VRs 1, 3, 5, 6, and 7 (three, two, nine, six, and three probes for each VR, respectively) were included for all assays. The hybridizations were performed as described previously (19). Briefly, 400 ng of denatured PCR-amplified *por* DNA was applied to Zeta-Probe-GT nylon membranes (Bio-Rad, Hercules, Calif.) by using a 30-slot vacuum apparatus (Immunetics, Cambridge, Mass.). Probes were hybridized by using a 45-channel vacuum apparatus (Immunetics) at 59°C for 3 h, and binding was visualized by using an avidin-peroxidase conjugate (Roche Molecular Biochemicals) and chemiluminescent substrate (ECL; Amersham Pharmacia Biotech, Piscataway, N.J.) followed by exposure to photographic film. Hybridization signals were assessed visually and compared to those of control strains.

Hybridization results for a single VR are referred to as "VR type," and in instances where more than one probe is hybridized for a single VR, each probe is listed separated by a comma. The *por* VR type includes results for all VRs tested, listed sequentially separated by a semicolon, e.g., PIA 1;2;1;1;1 denotes a strain hybridizing PIA VR1-1, VR2-2, VR3-1, VR6-1, and VR7-1 probes. Nt indicates an individual VR that failed to hybridize with the current panel of probes. A mixed *por* type was defined as hybridization of dissimilar probes (differing by ≥4 bp) at each of two or more individual VRs.

RESULTS

The goal of this study was to demonstrate the ability of our methods to amplify and characterize *porB* from a variety of experimental samples under different storage conditions and in the presence of more than one strain. We then used these methods to characterize gonococcal *por* VR types in available archived samples from two clinical studies.

TABLE 1. Effect of freeze-thaw cycle on PCR sensitivity in urine samples

Sample dilution	PCR product concn (ng/μl)[a]		
	Fresh	Frozen pellet	Frozen urine
10^{-1}	37	26	22
10^{-2}	10	+	4
10^{-3}	10	8	+
10^{-4}	4	1.5	+
10^{-5}	+	+	−

[a] A + indicates samples that generated a visible band below the limit of quantitation (1 ng/μl). A − indicates a sample that generated no visible band.

Sensitivity of nested *porB* PCR and effects of sample handling. The sensitivity of the nested PCR was determined for two PIB strains, MS11mkCe and FA1090, diluted into two different urine samples, one of which was shown to inhibit PCR. Sufficient product for subsequent *por* typing was generated in the nested PCR when 1 to 10 copies of template DNA were added to the PCR.

To determine the effect of a freeze-thaw cycle on the ability to amplify *porB* DNA from urine, we quantified the amplicon from PCR performed on DNA purified from urine spiked with serial dilutions of bacterial suspensions and stored under various conditions. Only the outer PCR was performed; bacterial suspensions ranged from approximately 10^6 to 10^2 CFU/ml. After a single freeze-thaw cycle, a 10-fold-higher concentration of bacteria was required to generate a detectable PCR product, and a 100-fold-higher concentration was necessary to yield a quantifiable amount of PCR product compared to a sample that had not been frozen. However, freezing the pellet alone after removing the supernatant did not result in a substantial loss of amplifiable DNA (Table 1).

***por* VR typing of mixed strains.** To examine the ability of *por* VR typing to identify more than one gonococcal isolate in a single sample, suspensions of a PIB isolate and a PIA isolate were mixed at various concentrations, DNA was purified and amplified, and *por* VR typing was performed. Purification, amplification, and *por* VR typing were achieved with as little as 10 to 10^2 copies of template DNA from either strain, even in the presence of a 100-fold excess of the other strain (Fig. 1).

Commensal *Neisseria* species. Genomic DNA from each of 13 *Neisseria* species was used as a template for *porB* amplification and VR probe hybridization. No PCR product was observed from either the outer or the nested reactions for *N. canis*, *N. cinerea*, *N. caviae*, *N. flavescens*, *N. mucosa*, *N. ovis*, *N. sicca*, and *N. weaveri*. The outer and/or nested PCR amplified a DNA fragment in the range of 871 to 1,192 bases (similar to the size of the expected product generated by amplification of gonococci) for *N. meningitidis*, *N. polysaccharea*, *N. lactamica*, *N. subflava*, and *N. elongata*. None of the PCR products obtained from nongonoccocal *Neisseria* species hybridized any of the gonococcal *por* VR probes.

***por* VR typing from matched cervical wick, swab, and culture samples.** Paired cervical swabs and cervical wicks from 20 women were examined, along with corresponding stored culture samples for 17 of these women. PCR product and typing results were obtained for all samples. Eight of 20 women had evidence of more than one *por* type. Multiple *por* types were

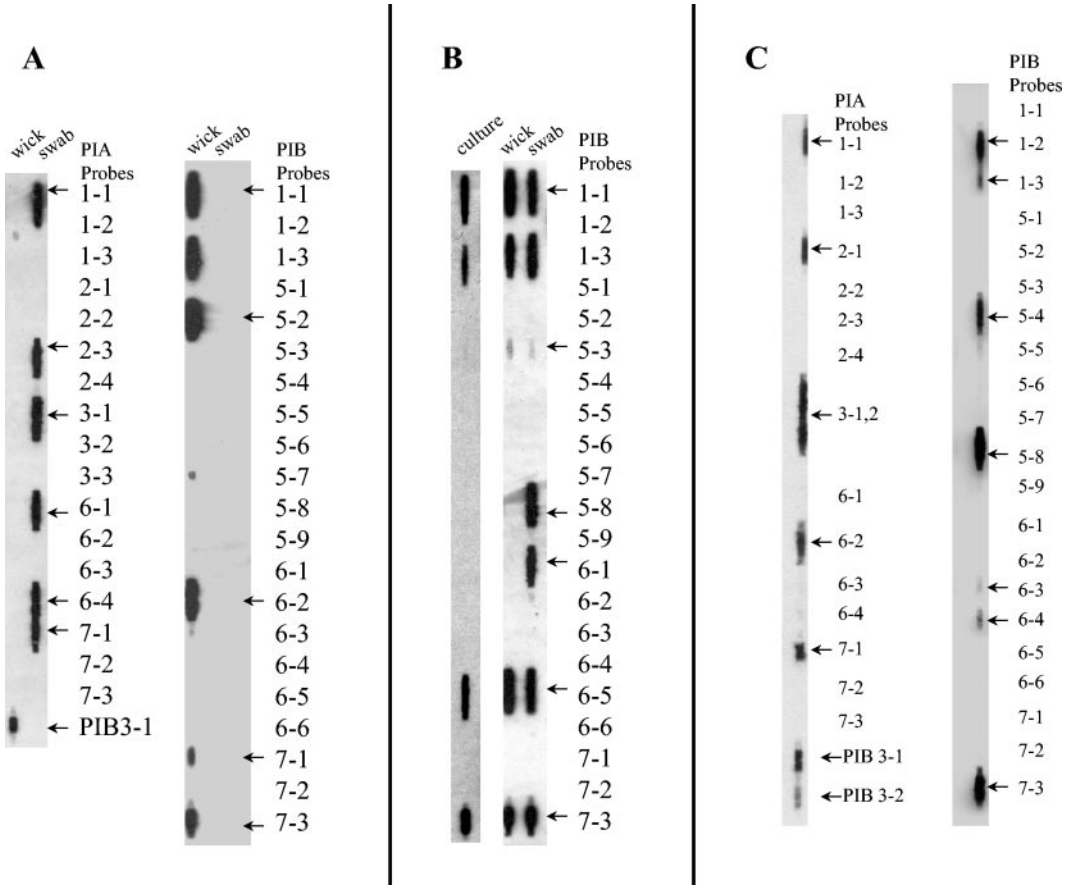

FIG. 2. Detection of multiple *por* VR types in clinical samples using checkerboard hybridization blots. PCR amplicons were applied to nylon membranes in columns, and probes were hybridized in rows. Arrows show hybridized probes that determine VR type. Where two sets of probes are indicated (panels A and C), the images are of the same blot, stripped and rehybridized. Shown are hybridization profiles of amplicons from samples from three women that demonstrate the presence of multiple *por* VR types. (A) Discordant cervical wick and cervical swab samples from a woman enrolled in Baltimore. The *por* VR type was PIB in the wick and was PIA in the swab. (B) Matched cervical wick, swab, and culture samples from a second woman enrolled in Baltimore. The culture, wick, and swab all contained one similar *por* VR profile; however, the swab sample bound additional probes (VR5-8 and VR6-1), suggesting the presence of a second strain with a different *por* type. Probes PIB VR5-3 and VR5-8 differ from each other by 11 bp, and PIB VR6-1 and VR6-5 differ by 10 bp; hybridization of these probes indicates the presence of two different PIB *porB* genes. (C) Hybridization pattern of *porB* PCR products from a single urine sample from a female SW enrolled in Madagascar. Hybridization of PIA probes, two PIB VR3 probes (right), two PIB VR1 probes, two PIB VR5 probes, and two PIB VR6 probes (left) suggests the presence of at least three *por* types. The PIB probes hybridized for VRs 1, 3, 5, and 6 differ by 10, 6, 3, and 6 bp, respectively. The hybridized PIA probes differed from PIB probes that bound to corresponding VRs by 6 to 12 bp.

detected from the wicks from four women, multiple *por* types were detected from the swabs from two women, and results from wicks and swabs from two women were discordant (Fig. 2A and B). In half (four of eight) of the samples, both a PIA and a PIB type were detected, and the remaining four samples had evidence of more than one PIB strain. No mixed *por* types were found in DNA purified from the stored culture samples; however, the *por* type identified in the DNA from each culture sample matched one of the *por* types identified in the corresponding cervical samples (Fig. 2B). The *por* type in the DNA from the culture sample matched the *por* type in the DNA from the swab in one of two discordant samples and matched the PIA strain in all three PIA/PIB mixed infections where a culture sample was available.

Overall, 18 *por* types were identified: 15 PIB types among 17 women and 3 PIA types among 6 women (4 who also had a PIB strain identified). One woman was infected with a PIA/PIB hybrid strain in which VR1 was typed as PIB and VRs 3, 6, and 7 were typed with PIA probes, suggesting a point of recombination between the regions encoding loops 2 and 3. Figure 3A shows the percentage of women with PIA, PIB, or mixed *por* types, and Fig. 4 shows the overall distribution of *por* types in the samples tested. To assess the reproducibility of our method, results for eight samples, including some mixed *por* VR types and the hybrid type, were confirmed by reamplification of the purified DNA and retyping.

***por* VR typing from urine samples obtained from female sex workers.** To explore the utility of *por* VR typing for clinical studies in which urine is often collected for sexually transmitted disease testing, we attempted to type gonococci from stored frozen urine collected from female SWs in Madagascar (2). Samples obtained at two visits approximately 2 months apart had been stored frozen for 2 to 2[1/2] years. The first 144 samples were subjected to an analysis blinded with respect to

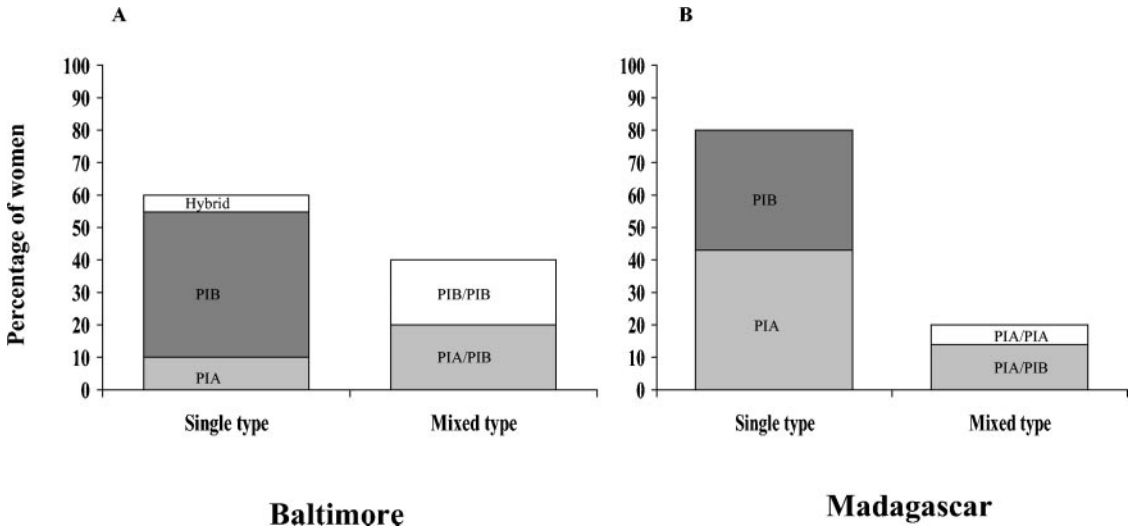

FIG. 3. Distribution of PIA, PIB, hybrid, and mixed *por* types. Percentages of women with PIA, PIB, hybrid, or mixed *por* types for each study are shown. (A) Women enrolled in the study in Baltimore for whom *por* type was determined; (B) women enrolled in the study in Madagascar for whom *por* type was determined.

LCR results, and amplification products were obtained from 48 of these 144 samples. *por* type was determined for 44 samples from 36 SWs: 25 of 53 LCR-positive (47%) and 1 of 19 LCR-negative (5%) samples from visit 1, and 16 of 42 LCR-positive (38%) and 2 of 30 LCR-negative (7%) samples from visit 2. The remaining four PCR-amplified samples did not hybridize any of the VR probes, consistent with the results from commensal *Neisseria* species. An additional 31 LCR-positive samples with larger available urine volumes (≥4 ml) were processed, and *por* type was determined for 8 of 15 samples from visit 1 (53%) and 11 of 16 samples from visit 2 (68%). In total, we amplified and typed *porB* in 63 samples from 53 SWs.

Thirteen of the 63 *por* VR-typed samples had results consistent with multiple *por* types (21%). Four samples indicated two PIA types, and two samples were from the two visits of a single SW. Coinfection with a PIA and a PIB strain was identified in nine samples, and two of these samples were from one woman. One of the PIA/PIB mixed samples had evidence of two different PIB *por* types, indicating the presence of DNA from at least three strains (Fig. 2C). In the remaining typed samples, 23 (37%) had a single PIB *por* type and 27 (43%) had a single PIA *por* type (Fig. 3B and 4).

Overall, 10 PIB *por* types were identified among 32 samples from 28 SWs, and three types accounted for 23 (72%) of the samples. Ten PIA *por* types were identified among 40 samples from 35 SWs, and three types accounted for 26 (65%) of the samples. PCR-positive and typeable results were obtained from urine at both the first and second visits for 10 subjects. Of these samples, four had the same *por* type or types at both visits, three had completely different *por* types at each visit, and three had the same *por* type at both visits but also had a second *por* type at one of the two visits. To assess the reproducibility of our typing method, results for 12 samples, including the sample with evidence of three different *por* VR types, were confirmed by reamplification of the purified DNA and retyping.

Comparison of *por* VR typing in Baltimore and Madagascar samples. Calculation of the frequency of individual *por* types was complicated by the presence of mixed *por* types. Mixed PIA and PIB types were treated as individual PIA and PIB *por* types, and three of four individuals from Baltimore with more than one PIB type could be resolved into separate types by comparisons among cervical and culture samples. Five suspected coinfections with more than one PIA strain identified in urine samples were each treated as unique combined *por* types. This approach may have underestimated the frequency of the composite types and overestimated the diversity of PIA *por* types, but an overall picture of *porB* diversity within and among the sample sets was still possible (Fig. 4). Several *por* types were identified in both sets of samples described here (Table 2).

Analysis of individual VRs indicated that the panel of VR probes used in this study was highly relevant to both sets of samples. Among 63 Madagascar samples, one common PIB strain was nontypeable in VR5, one PIA sample was nontypeable in VR1, and another PIA sample was not typed in VR1 and VR2. In the Baltimore samples, one PIB strain was nontypeable in VR5 and VR7.

DISCUSSION

This study expands our earlier work with *por* VR typing of culture samples by applying this method to noncultured clinical samples. Adapting the method for use with direct clinical samples allowed us to investigate the possible occurrence of coinfection with multiple gonococcal strains in more detail than previously reported as well as to examine the range of *por* types identified in two very divergent populations.

The use of NAAT in conjunction with the ability to characterize strains from direct clinical samples may have an important impact on the study of *N. gonorrhoeae*. Turner et al. (28) estimated that the prevalence of undiagnosed gonococcal infections in a young adult population in Baltimore was 5.3% by

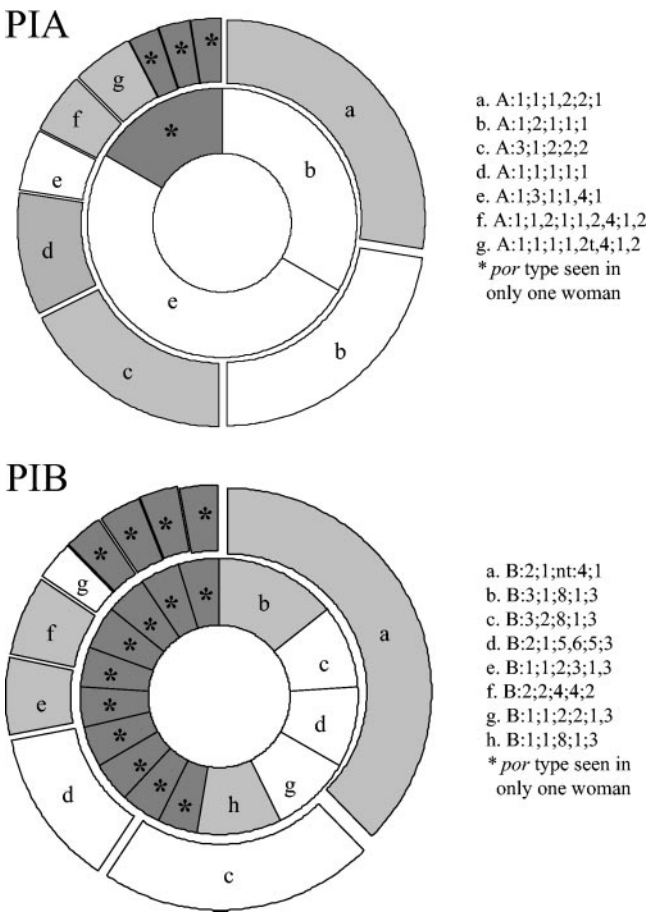

FIG. 4. Distribution of *por* types from study samples for which typing data were obtained. The inner circle shows *por* types identified from Baltimore, and the outer circle shows those identified from Madagascar. Women with mixed PIA/PIB *por* types contribute to the totals for each *por* type. Mixed PIA/PIA *por* types were treated as a unique PIA type, and mixed PIB/PIB types that could not be separately identified were counted as a unique PIB type. A total of 6 women had 1 of 3 PIA *por* types and 17 women had 1 of 15 PIB types in Baltimore; a total of 35 women had 1 of 10 PIA types and 28 women had 1 of 10 PIB types in Madagascar. The most commonly identified *por* types are listed by lowercase letters corresponding to the labeled graphic. The *por* types that were observed in both geographic locations are in white. The *por* types unique to each group are shaded. *por* types occurring only once are indicated by an asterisk.

TABLE 2. *por* types common to women in Baltimore and Madagascar

Allele	*por* type[a]	% of women with *por* type[b]	
		Baltimore	Madagascar
PIA	A:1;2;1;1;1 (b)	10	17
	A:1;3;1;1,4;1 (e)	15	4
Total % of women with PIA types observed in both locations		25	21
PIB	B:3;2;8;1;3 (c)	10	13
	B:1;1;2;2;1,3 (g)	10	2
	B:2;1;5,6;5;3 (d)	10	8
Total % of women with PIB types observed in both locations		30	23
Total % of women with *por* types observed in both locations		55	44

[a] The *por* VR type includes results for all VRs tested listed sequentially separated by a semicolon; where more than one probe is hybridized for a single VR, each probe listed is separated by a comma. Letters in parentheses designate legend for that strain in Fig. 4.

[b] The percentages are based on the total number of women for whom typing data were obtained.

using NAAT, much higher than the 2.6% prevalence estimate based on diagnosed and reported infections in the same population. In Australia, a study using NAAT found that the estimated number of women infected was over four times the number of officially reported cases (3). The characteristics of isolates causing infections identified by NAAT have not been routinely examined, and epidemiologic studies utilizing NAAT will be enhanced by the use of methods that provide molecular characterization of strains.

In addition to potential application with current NAAT, *por* VR typing is based on characterization of a single-copy gene and is therefore able to distinguish single, multiple, or hybrid *por* types present in a single sample. Our previous data demonstrated that the oligonucleotide probes do not cross-react with sequences that differ by more than 2 bp and are highly specific for the sequences found in the variable regions of *porB* (19, 27). Sequence analysis of neisserial *porB* genes has shown that while genetic exchange occurs between genes, exchange does not occur between VRs within a single *porB* gene (1, 9, 12, 24, 27, 29, 33). For example, sequences found in VR1 are unique to that VR and are not found in any other VR in the gene. Given the specificity of the oligonucleotide probes, hybridization of distinctly different probes for the same VR region strongly suggests that DNA from more than one strain was present in the sample. Mixed gonococcal infections may be significant in many aspects of gonococcal research, including studies of natural immunity, transmission, spread of antimicrobial resistance, and genetic diversification. The extensive mechanisms present in *N. gonorrhoeae* for genetic exchange and recombination have been recently reviewed (13). Since the only ecologic niche occupied by gonococci is the human host, mixed infections are central to the evolution of this pathogen.

Typing directly from clinical specimens identified multiple *por* types from a remarkable 40% of the women in the Baltimore study and from 22% of specimens from SWs from Madagascar. While the specimens that were used for this exploratory study do not allow estimates of the prevalence of mixed infections in any population, the data suggest that mixed infections are far more common than previously appreciated. Knapp et al. had previously reported a rate of approximately 21% when isolating *N. gonorrhoeae* from multiple sites in men and women and identifying different auxotype/serovar classes from each site (14). However, the identification of multiple auxotype/serovar types in a single mixed isolate would be difficult, as the serovar system uses a panel of monoclonal antibodies and unusual binding patterns may be due to either usual serovars

(such as those seen in hybrid or rare porin types) or mixed infections. Martin and Ison (18) recently raised this issue by comparing the *opa* type of direct urethral specimens with the *opa* type of the primary culture. In that study, 19 samples (14 from men and 5 from women) were examined, and four men (21%) had evidence of a mixed gonococcal infection. Those authors did not detect multiple strains in cultures, even by typing multiple individual colonies of the primary isolation plate. In our study, DNA purified from stored culture samples also did not contain evidence of multiple strain types. We did not attempt to identify *por* types of single colonies grown from the culture samples, as we avoided possible secondary selection by purifying DNA directly from an aliquot of the stock prepared from the primary culture plate. Since amplification and identification of one *porB* in the presence of a 100-fold excess of another *porB* was possible, this approach should be more sensitive than screening several hundred colonies.

The discrepancy between the ability to detect mixed infections in stored cultures and the ability to detect mixed infections in stored clinical specimens could have several plausible explanations. Nonviable and live gonococci would be detected by DNA amplification from clinical samples, whereas only viable gonococci would be recovered by culture. Differences in in vitro growth kinetics might bias detection towards more robust strains that grow well on standard culture media. Even if two strains were present on a culture plate, they would not necessarily be distinguishable by colony morphology, and multiple single-colony analyses would be necessary to identify and characterize both strains. So although not a factor in this study, isolation of a single colony to characterize an infecting strain may have underestimated mixed infections in previous studies. The use of NAAT diagnostics combined with molecular methods of strain characterization may greatly improve our understanding of the frequency and consequences of mixed gonococcal infections.

Although comparisons of the diversity of *por* types between Baltimore and Madagascar are limited by the differences in study design, some general observations can be made. A high proportion of individuals for whom typing data were obtained had a *por* type common to both geographic areas. Our earlier study of Baltimore isolates collected over 10 years (19) suggested that approximately 10 to 33% of cultures were PIA, which is consistent with the findings of the present study as well as those of others (14). The unusually high number of PIA strains in the Madagascar SWs, in which 66% of women had at least one PIA strain, may be a consequence of factors including the number of exposures, general physical condition, and immune status. The differential ability of PIA compared to PIB strains to be isolated outside the genital tract, survive in the harsh environment of urine, and remain intact during storage and handling may affect the detection of different *por* types. Additionally, we were not able to amplify and type all of the samples that were LCR positive, and we cannot exclude the possibility of sample bias affecting the relative prevalence of PIA- compared to PIB-expressing strains observed in this study. However, given that more serious, invasive, and disseminated disease has been associated with strains expressing PIA, the predominance of PIA in this population deserves further investigation.

The PCR primers used in this study were specifically designed to amplify both PIA and PIB alleles of *porB*. Due to the conserved nature of the of neisserial porins, the primers also amplified DNA from some commensal *Neisseria* species. Other NAAT systems also detect nongonococcal genes (8, 20, 21, 31). Our data suggest, however, that gonococcal *porB* probes do not hybridize to porin DNA amplified from commensal *Neisseria*, allowing accurate characterization of the gonococcal porin even in the presence of commensal *Neisseria*.

We were unable to amplify *porB* from many of the LCR-positive urine samples. Experimentally, we demonstrated the loss of an order of magnitude in sensitivity associated with a single, brief freeze-thaw cycle of whole urine, and additional losses may be associated with extended storage time. The Abbott GC LCR targeted the multicopy gene *opa* rather than the single-copy gene *porB* targeted by *por* VR typing. LCR may be more sensitive than PCR; no PCR-based NAAT is widely available for the detection of gonococci in urine from women. *por* VR typing is not designed to be a diagnostic test per se but rather a method that can be used in conjunction with NAAT to characterize strains in individuals who have been identified as having gonococcal infections. Several studies have shown that endocervical and vaginal samples are more sensitive than urine for detecting gonococci in women (6, 15, 17, 23, 26), and the use of self-collected vaginal swabs might be a viable alternative (17, 26). In studies where urine samples are collected, centrifuging the samples and storing the pellets alone may improve subsequent genotypic analyses.

In these studies, we demonstrated the utility of molecular characterization of the gonococcal outer membrane Por protein from non-culture-based clinical samples. We have also shown that evidence of infections with multiple strains can be easily identified in direct clinical specimens. Additionally, our data support our earlier findings that some *por* types and some VR types are common among geographically diverse populations. The combination of a molecular typing method with NAAT will enhance our understanding of the molecular epidemiology of *N. gonorrhoeae*. Our understanding of the significance of gonococcal *por* type and mixed infections in studies of protective immunity, strain transmission, reinfection, and antimicrobial resistance will be substantially enhanced by using direct clinical specimens and methods that provide specific and accurate information about *porB*.

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